Docket No. 210352US0X

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Ryuichiro KURANE, et al.

SERIAL NO. NEW APPLICATION

FILING DATE: HEREWITH

FOR: NOVEL NUCLEIC ACID PROBES, METHOD FOR DETERMINING CONCENTRATIONS OF NUCLEIC ACID BY USING THE PROBES, AND METHOD FOR ANALYZING DATA OBTAINED BY THE METHOD

## STATEMENT RE FILING IN FOREIGN LANGUAGE

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

It is hereby stated that the subject application is partly being filed in a foreign language, in accordance with the provisions of 37 CFR 1.52(d).

An accurate English translation, and a suitable amendment placing the application and claims thereof into proper U.S. format if needed, will be filed in due course.

Respectfully Submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon
Registration No. 24,618

22850
Tel. (703) 413-3000
Fax. (703) 413-2220
(OSMMN 10/98)

#10

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| IN RE APPLICATION OF: | : | |
| Ryuichiro KURANE, et al. | : | |
| SERIAL NO: 09/891,517 | : | ATTN: BOX MISSING PART |
| FILED: June 27, 2001 | : | |
| FOR: NOVEL NUCLEIC ACID PROBES, METHOD FOR DETERMINING CONCENTRATIONS OF NUCLEIC ACID BY USING THE PROBES, AND METHOD FOR ANALYZING DATA OBTAINED BY THE METHOD | : | |

**FILING OF CERTIFIED ENGLISH TRANSLATION UNDER 37 CFR 1.52(d)**

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Notice to File Missing Parts of Application (Form PTO-1533) dated July 17, 2001, Applicants submit herewith a certified English translation of example 17 to example 42 in the specification , as filed, in accordance with the provisions of 37 C.F.R. §1.52(d).

The required fee was paid at the time of filing of the application.

In light of the foregoing, this application is deemed to be in proper condition for examination and such favorable action is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518

22850
Tel. (703) 413-3000
Fax. (703) 413-2220

DECLARATION

I, Tadashi Tsukamoto of 41-8, Utsukushigaoka 3-chome, Aoba-ku, Yokohama, Kanagawa 225-0002, Japan do solemnly and sincerely declare that I well understand both Japanese and English languages.

The translation attached hereto is a true and accurate translation of pages 133 through 180 (Examples 17 through 42) of U.S. patent application Serial No. 09/891,517 which were filed in Japanese.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

This 8th day of September, 2001

Tadashi Tsukamoto

Example 17 (Experiment on contribution of 2'-O-Me-oligonucleotide and helper probe to the efficiency of hybridization)

Various invention probes and helper probes, which were to be hybridized to the above-described 16S rRNA, were prepared in a similar manner as in Example 16. The 2-O-Me-oligonucleotides for use in 2-O-Me probes were all obtained by ordering their synthesis to GENSET SA, Paris, France. Under conditions to be described subsequently herein, an investigation was made about effects of the 2'-O-Me probes of the present invention, effects of the lengths of nucleotide chains in the probes and effects of helper probes in the experiment groups of diagrams A, B, C and D in FIG. 9 in a similar manner as in Example 16. The results are presented in FIG. 9.

It is appreciated from these diagrams that the 2-O-Me probes according to the present invention contribute to the efficiency of hybridization. It is also understood that these helper probes are effective in increasing the efficiency of hybridization when the base strands of the 2-O-Me probes are short.

1) 35-Nucleotides-chained 2-O-Me probe: Same probe as in Example 16.

2) 35-Nucleotides-chained DNA probe: A probe having the same base sequence as the 35-nucleotides-chained 2-O-Me probe described above under 1) except that the oligonucleotide is

formed of a deoxyribose.

3) 17-Nucleotides-chained 2-O-Me probe: A probe having the same base sequence as the 35-nucleotides chained 2-O-Me probe described above under 1) except that the nucleotides ranging over 8 bases from the 5′ end and 10 bases from the 3′end were removed.

4) 17-Nucleotides-chained DNA probe: A probe having the same base sequence as the 33-nucleotides-chained DNA probe described above under 2) except that a nucleotide ranging over 16 bases from the 3′end was removed.

5) Forward-type 2-O-Me-helper probe: A helper probe obtained by modifying (via an ether bond) the OH group on the carbon atom at the 2′-position of ribose over the central 8 bases (the $9^{th}$ base to the $16^{th}$ base counted from the 5′end) of the forward-type helper probe in Example 16 with a methyl group.

6) Reverse-type 2-O-Me-helper probe: A helper probe obtained by modifying (via an ether bond) the OH group on the carbon atom at the 2′-position of ribose over the central 8 bases (the $9^{th}$ base to the $16^{th}$ base counted from the 5′end) of the reverse-type helper probe in Example 16 with a methyl group.

7) Forward-type DNA helper probe: A helper probe having the same base sequence as the forward-type helper probe in Example 16 except that the oligonucleotide is formed of a deoxyribonucleotide.

8) Reverse-type DNA helper probe: A helper probe having

the same base sequence as the reverse-type helper probe in Example 16 except that the oligonucleotide is formed of a deoxyribonucleotide.

9) 35-Base oligoribonucleotide: An oligoribonucleotide having a base sequence of (5′)CATCCCCACC TTCCTCCGAG TTGACCCCGG CAGTC(3′).

10) 17-Base oligoribonucleotide: An oligoribonucleotide having a base sequence of (5′)CCTTCCTCCG AGTTGAC(3′).

Reaction conditions:

| | |
|---|---|
| Concentration of 16S rRNA: | 10 nM |
| Concentration of probe: | 25 nM |
| Helper probe concentration: | 1 $\mu$M |
| Buffer composition: | 100 mM succinic acid, 125 mM lithium hydroxide, 8.5% lithium dodecyl-sulfite, pH 5.1 |

Reaction temperature:

75℃ (for 35-nucleotides-chained 2-O-Me probe)

70℃ (for 17-nucleotides-chained 2-O-Me probe)

75℃ (for 33-nucleotides-chained DNA probe)

60℃ (for 17-nucleotides-chained oligoribo-nucleotide probe)

Experiment system, FIG. 9A:

$HP(M)^+$: 16S rRNA, 35-nucleotides-chained DNA probe, forward-type 2-O-Me helper probe, reverse-type

2-O-Me helper probe,

HP(D)$^+$: 16S rRNA, 35-nucleotides-chained DNA probe, forward-type DNA helper probe, reverse-type DNA probe,

HP-: 16S rRNA, 35-nucleotides-chained DNA probe, and

Ref (Control): 35-nucleotides-chained DNA oligoribonucleotide, 35-nucleotides-chain.

Experiment system, FIG. 9B:

HP(M)$^+$: 16S rRNA, 35-nucleotides-chained 2-O-Me probe, forward-type 2-O-Me helper probe, reverse-type 2-O-Me helper probe,

HP(D)$^+$: 16S rRNA, 35-nucleotides-chained 2-O-Me probe, forward-type DNA helper probe, reverse-type DNA probe,

HP-: 16S rRNA, 35-nucleotides-chained 2-O-Me probe, and

Ref (Control): 35-nucleotides-chained DNA oligoribonucleotide, 35-nucleotides-chained 2-O-Me probe.

Experiment system, FIG. 9C:

HP+(M): 16S rRNA, 17-nucleotides-chained DNA probe, forward-type 2-O-Me helper probe, reverse-type 2-O-Me helper probe,

HP+(D): 16S rRNA, 17-nucleotides-chained DNA probe, forward-type DNA helper probe, reverse-type DNA

probe,

HP-: 16S rRNA, 17-nucleotides-chained DNA probe, and

Ref (Control): 17-nucleotides-chained DNA oligoribonucleotide, 17-nucleotides-chain.

Experiment system, FIG. 9D:

HP+(M): 16S rRNA, 17-nucleotides-chained 2-O-Me probe, forward-type 2-O-Me helper probe, reverse-type 2-O-Me helper probe,

HP+(D): 16S rRNA, 17-nucleotides-chained 2-O-Me probe, forward-type DNA helper probe, reverse-type DNA probe,

HP-: 16S rRNA, 17-nucleotides-chained 2-O-Me probe, and

Ref (Control): 17-nucleotides-chained DNA oligoribonucleotide, 17-nucleotides-chained 2-O-Me probe.

Example 18

(Preparation of working curve for rRNA determination)

At diverse concentrations within a range of from 0.1 to 10 nM, the above-described rRNA was heated at 95℃ for 5 minutes. The resulting nucleic acid solutions were added to aliquots of a reaction mixture, respectively. The reaction mixture had been prepared and maintained under the below-described reaction conditions. Upon elapsed time of 1,000 seconds, decreases in fluorescence intensity were measured using "Perkin-Elmer

LS-50B". The results are plotted in FIG. 10. It is appreciated from the diagram that the working curve shows linearity in the range of from 0.1 to 10 nM. Incidentally, the following 35-nucleotides-chained 2-O-Me probe was the same probe as that prepared in Example 16.

Reaction conditions:

| | |
|---|---|
| Concentrations of 35-nucleotides-chained 2-O-Me probe: | 1.0 to 25 nM |
| Buffer composition: | 100 mM succinic acid, 125 mM lithium hydroxide, 8.5% lithium dodecyl-sulfite, pH 5.1 |
| Reaction temperature: | 75℃ |

Example 19

(FISH method)

In a similar manner as described above, the below-described 35- and 36-nucleotides-chained oligodeoxyribonucleotide 2-O-Me probes according to the present invention were prepared for hybridization to the respective rRNAs of *Cellulomonas sp.* KYM-7 (FERM P-11339) and *Agrobacterium sp.* KYM-8 (FERM P-16806), respectively. Those probes had the following base sequences:

35-nucleotides-chained oligodeoxyribonucleotide 2-O-Me probe for assaying the rRNA of *Cellulomonas sp.* KYM-7:

(5')CATCCCCACC TTCCTCCGAG TTGACCCCGG CAGTC(3')

(the underlined portion is modified with a methyl group)

36-nucleotides-chained oligodeoxyribonucleotide 2-O-Me probe for assaying the rRNA of *Agrobacterium sp.* KYM-8:

(5′)CATCCCCACC TTCCTCTCGG CTTATCACCG GCAGTC(3′)

(the underlined portion is modified with a methyl group)

*Cellulomonas sp.* KYM-7 and *Agrobacterium sp.* KYM-8 were co-cultured on a culture medium of the below-described composition under the below-described cultivation conditions. Co-cultures were sampled at various phases of the co-cultivation. From each of the co-cultures, rRNAs were prepared using "RNeasy Maxikit" (trade name; product of QIAGEN GmbH (Hilden, Germany). Those rRNAs were heated at 95℃ for 5 minutes, and then added to the reaction mixture which had been maintained under the reaction conditions. After they were reacted at 70℃ for 1,000 seconds, the intensity of fluorescence was measured using "Perkin-Elmer LS-50B". The results are plotted in FIG. 11. Incidentally, the total rRNA was measured using "RiboGreen Total RNA Quantification Kit" [trade name; product of Molecular Probe, Inc. (Eugene, Oregon, U.S.A.)].

As is appreciated from the diagram, the mobilizations of the rRNAs of the respective cell strains were consistent with that of the total rRNA. This indicates that the method of the present invention can be effectively used in the FISH method.

Composition of culture medium (g/L):

Starch, 10.0; aspartic acid, 0.1 ; $K_2HPO_4$, 5.0; $KH_2PO_4$,

2.0; $MgSO_4$ $7H_2O$, 0.2; NaCl, 0.1; $(NH_4)_2SO_4$, 0.1. Aliquots (100 mL, each) of the culture medium were poured in 500-mL Erlenmeyer flasks, and were sterilized in an autoclave at 120℃ for 10 minutes.

Cultivation conditions:

The above-described cell strains were cultivated beforehand on a slant medium. One roopful of cells was collected from the slant medium, and was then inoculated to the above-described sterilized nutrient broth (NB) in the Erlenmeyer flask. The strains were cultured at 30℃ and 150 rpm under shaking.

Reaction conditions:

| | |
|---|---|
| Concentrations of 35-nucleotides-chained oligodeoxyribonucleotide | |
| 2-O-Me probe: | 1.0 to 10 nM |
| Buffer composition: | 100 mM succinic acid, 125 mM lithium hydroxide, 8.5% lithium dodecyl-sulfite, pH 5.1 |
| Reaction temperature: | 75℃ |

Example 20

(Example directed to intra-chain modified fluorescence quenching probes)

Target nucleic acids and invention nucleic acid probes, which had the below-described base sequences, were prepared.

To provide probes a),b), amino linkers were introduced into the corresponding base sequences by using "Amino-Modifier C6 dC" (trade name, product of Glen Research Corporation, VA, U.S.A.), and the amino linkers were labeled with BODIPY FL. Except for these, the probes a),b) were synthesized in a similar manner as in Example 8. Therefore, the probe a) was modified with the fluorescent dye on the C base at the 5'end rather than the phosphate group at the 5'end. Modification with BODIPY FL, purification and the like were conducted in a similar manner as described above.

Probe a): 5'C(-BODIPY FL)TTTTTTTTCCCCCCCCC3'

Probe b): 5'TTTC(-BODIPY FL)TTTTTTCCCCCCCCC3'

Target nucleic acid c) for Probe a):

5'GGGGGGGGAAAAAAAAAG3'

Target nucleic acid d) for Probe b):

5'GGGGGGGGAAAAAAGAAA3'

<Experimenting method>

An experiment was conducted in a similar manner as in Example 9.

<Results of the experiment>

As is readily envisaged from the table described below, it has been found that the probe a) and the probe b) are both reduced in the intensity of fluorescence when they hybridize to the corresponding target nucleic acids. It has also been found from the results on the probe b) that modification of a

cytosine base in a DNA chain at a position other than the 5'end or 3'end with a fluorescent dye also permits functioning as a fluorescence quenching probe. It has also been found from the results on the probe a) that, even in the case of an end cytosine, modification at a position other than the phosphate group at the 5'end or the OH group at the 3'end with a fluorescent dye makes it possible to obtain a fluorescence quenching probe.

Table 6

Results of Example 20

| | Intensity of fluorescence before hybridization | Intensity of fluorescence after hybridization | Quenching rate of fluorescence (%) |
|---|---|---|---|
| Probe a) + Target nucleic acid c) | 410 | 75 | 81.7 |
| Probe b) + Target nucleic acid d) | 380 | 82 | 78.4 |

A method for analyzing or determining polymorphism and mutation of target nucleic acids will hereinafter be described in Example 21.

Example 21

Four oligonucleotides with the below-described base sequences were synthesized using the same DNA synthesizer as that employed in Example 12. Further, an invention nucleic acid probe having the below-described base sequence was also

synthesized in a similar manner as in Example 12. The oligonucleotides were separately hybridized with the probe in solutions. An investigation was then made as to whether or not a single base substitution can be determined from a change in fluorescence intensity. The base sequence of the nucleic acid probe according to the present invention is designed such that, if G exists at the 3'end of any one of the target oligonucleotides, it matches 100% with the base sequence of the particular oligonucleotide. The hybridization temperature was set at 40℃ at which all base pairs between the probe and the target oligonucleotide can hybridize 100%. The concentrations of the probe and target oligonucleotides, the concentration of a buffer solution, a fluorimeter, fluorescence measuring conditions, experimental procedures, and the like were set or chosen as in Example 12.

Invention probe: 3'TTTTTTTTGGGGGGGGC5'BODIPY FL/C6

Target nucleotide No. 1: 5'AAAAAAAACCCCCCCCA3'

Target nucleotide No. 2: 5'AAAAAAAACCCCCCCCC3'

Target nucleotide No. 3: 5'AAAAAAAACCCCCCCCI3'

(I: hypoxanthine)

Target nucleotide No. 4: 5'AAAAAAAACCCCCCCCG3'

The results are shown in Table 7. As is appreciated from the table, no change in fluorescence intensity was observed in the case of the target oligonucleotides Nos. 1 to 3, but in the case of the target oligonucleotide No. 4, a decrease as much

as 84% was observed.

Table 7

| Target oligo-nucleotide | Initial fluorescence intensity (A) | Fluorescence intensity after hybridization (B) | (A-B)/B |
|---|---|---|---|
| No. 1 | 340 | 350 | -0.03 |
| No. 2 | 332 | 328 | 0.01 |
| No. 3 | 343 | 336 | 0.02 |
| No. 4 | 345 | 52 | 0.84 |

In the method of the present invention for analyzing data (for example, the data in columns A and B in Table 7) obtained by the method for analyzing or determining polymorphism and/or mutation of a target nucleic acid (for example, the target oligonucleotide No. 1, 2, 3 or 4), the processing to correct a fluorescence intensity of a reaction system, said fluorescence intensity being obtained when a target nucleic acid is hybridized with a nucleic acid probe according to the present invention (for example, the above-described nucleic acid probe), by a fluorescence intensity of the same reaction system when the target nucleic acid is not hybridized with the nucleic acid probe means the calculation of (A-B)/B in Table 4.

From the above results, it has been found that, when a

target nucleic acid is a double-stranded nucleic acid, substitutions of G→A, G←A, C→T, C←T, G→C and G←C can be detected.

Example 22

One example of a DNA chip model according to the present invention is illustrated in FIG. 12. Firstly, a modified probe and a surface-treated slide glass were provided. The modified probe had been prepared by introducing an amino group onto the OH group on the carbon atom at the 3' position of ribose at the 3'end of the invention probe, 3'TTTTTTTTGGGGGGGGC5'BODIPY FL/C6, prepared in Example 21. On the other hand, the surface-treated slide glass had been prepared by treating a slide glass on a surface thereof with a silane coupling agent which contained epoxy groups as reactive groups. A solution with the modified probe contained therein was applied in spots onto the surface-treated slide glass by a DNA chip production apparatus, "GMS™ 417 ARRAYER" (manufactured by TAKARA SHUZO CO., LTD., Kyoto, Japan). As a result, the modified probes were bound at the 3'end onto the surface of the slide glass. The slide glass was then placed for 4 hours or so in a closed vessel to bring the reaction to completion. The slide glass was alternately dipped in 0.2% SDS solution and water, twice in each of the solution and water, for about 1 minute each time. Further, the slide glass was immersed for about 5 minutes in a boron solution, which had been prepared by dissolving $NaBH_4$ (1.0 g)

in water (300 mL). Shortly after the slide glass was placed for 2 minutes in water of 95℃, the slide glass was alternately dipped in 0.2% SDS solution and water, twice in each of the solution and water, for about 1 minute each time, so that reagents were washed off. The slide glass was then dried. As a result, a DNA chip according to the present invention was prepared.

Further, arrangement of a minute temperature sensor and a microheater on the lower side of the slide glass at a position corresponding to each spot of the modified probe makes it possible to provide the DNA chip of the present invention with high performance.

A description will next be made of determination of a target nucleic acid by the DNA chip. No change takes place in fluorescence intensity where the target nucleic acid is not hybridized with the probe, where no G-C pair is formed at the fluorescent-dye-labeled end, or where at least one G (guanine) or C (cytosine) base does not exist in the base sequence of the target nucleic acid at a position 1 to 3 bases from an end base portion where the probe and the target nucleic acid are hybridized with each other. However, the intensity of fluorescence decreases conversely where the target nucleic acid is hybridized with the probe, where a G-C pair is formed at the fluorescent-dye-labeled end even if they are hybridized together, or where at least one G (guanine) or C (cytosine) base

exists in the base sequence of the target nucleic acid at a position 1 to 3 bases from an end base portion where the probe and the target nucleic acid are hybridized with each other. This fluorescence intensity can be measured by using a DNA chip analyzer, "GMS™ 418 Array Scanner" (manufactured by Takara Shuzo Co., Ltd., Kyoto, Japan).

Example 23

[Experimental detection of single nucleotide polymorphism (SNPs)]

I) Preparation of target nucleic acid

An oligodeoxyribonucleotide having the base sequence of (5')AAACGATGTG GGAAGGCCCA GACAGCCAGG ATGTTGGCTT AGAAGCAGCC(3') was synthesized using a DNA synthesizer "ABI 394" (trade name; manufactured by Perkin-Elmer Inc., MA, U.S.A.), and was provided as a target nucleic acid.

II) Preparation of nucleic acid probes

The following six oligodeoxyribonucleotides, which had base sequences hybridizable to a sequence of 15 bases (underlined portion) from the 5'end of the target nucleic acid, were synthesized using the DNA synthesizer "ABI 394" (trade name; manufactured by Perkin-Elmer Inc., MA, U.S.A.). Using "3'-Amino-Modifier CY CPG" (trade name, product of Glen Research Corporation, VA, U.S.A, Catalog No. 20-2957), the OH group at the 3'-position of deoxyribose at the 3'end was aminated. Further, the phosphate group at the 5'end was labeled

with BODIPY FL in a similar manner as in Example 12.

1) Probe 100 (100% matched):

(5')CCTTCCCACA TCGTTT(3'),

2) Probe-T (1 base mismatched):

(5')CCTTCCCATA TCGTTT(3'),

3) Probe-A (1 base mismatched):

(5')CCTTCCCAAA TCGTTT(3'),

4) Probe-G (1 base mismtached):

(5')CCTTCCCAGA TCGTTT(3'),

5) Probe-TG (2 bases mismatched):

(5')CCTTCCCTGA TCGTTT(3'), and

6) Probe-TGT (3 bases mismatched):

(5')CCTTCCCTGT TCGTTT(3').

III) Preparation of DNA chip

All the DNA probes were dissolved in aliquots of 0.1 M MES (2-morpholinoethanesulfonic acid) buffer (pH 6.5) to give solutions of 500 nM in concentration. Using a DNA microarrayer [a manual chip arrayer composed of "DNA Microarrayer No. 439702" (32-pin type) and "DNA Slide Index No. 439701"; manufactured by Greiner GmbH, Frickerhausen, Germany], the above-described probe solutions were applied in spots onto a DNA chip slide glass (black silylated slide, product of Greiner GmbH, Frickerhausen, Germany). Subsequent to completion of the application in spots, the DNA probes and the slide glass were reacted for 60 minutes at room temperature in a humidity chamber to fix the probes on the slide glass. The slide glass with the DNA probes fixed

thereon was then washed with 50 mM TE buffer (pH 7.2). Incidentally, the probe solutions were applied four spots by four spots, respectively. Subsequent to the fixing, the slide glass was washed once with 0.1% SDS (sodium dodecylsulfate), washed twice with distilled water, and then immersed for 5 minutes in a solution of sodium borohydrate (2.5 mg $NaBH_4$/mL-25% ethanol solution). The slide glass was immersed for 3 minutes in a water batch heated at 95℃, and then dried.

A schematic illustration of the DNA chip according to the present invention is shown in FIG. 12. In each probe of the present invention fixed on the slide glass, BODIPY FL develops its color when the probe is not hybridized with a target nucleic acid but, when it is hybridized, its color development is less, namely, reduced than the color developed when it is not hybridized. The slide glass is designed to be heated by microheaters [in the present invention, the experiment was conducted on a transparent warming plate for microscope ("MP-10MH-PG", trade name; product of KITAZATO SUPPLY Co., Ltd., Shizuoka, Japan) as will be described below].

IV) Detection or determination of SNPs

A target nucleic acid solution of 100 μM in concentration [50 mM TE buffer (pH 7.2) was used] was placed on the DNA chip prepared as described above. A cover glass was placed over the solution, and was sealed with a nail varnish to avoid leakage of the target nucleic acid. A schematic illustration of

equipment for detection or determination is shown in FIG. 13. Firstly, a transparent warming plate for microscope ("MP-10MH-PG", trade name; manufactured by KITAZATO SUPPLY Co., Ltd., Shizuoka, Japan) was placed on a stage of an Olympus erect focal laser microscope (Model: AX80). The DNA chip according to the present invention, which had been prepared as described above, was placed on the plate, and the temperature of the plate was changed 3℃ by 3℃ from 95℃ to 33℃ such that the target nucleic acid and the probes were reacted for 30 minutes. Changes in the intensity of fluorescence at each spot in the course of the reaction were measured in an image-inputting manner by a cooled CCD camera ("C4880-40 Model", trade name; manufactured by Hamamatsu Photonics K.K., Shizuoka, Japan).

Inputted images were analyzed by an image analyzer [specifically, an NEC personal computer with image analysis software ("TPlab Spectrum", trade name; available from Signal Analytics, VA, U.S.A.) installed therein] to calculate the luminance values of the individual spots and further to determine a temperature-luminance relationship.

The results of the experiment are diagrammatically shown in FIG. 14. It is appreciated from the diagram that the intensity of luminance was decreased in all the probes. The method of the present invention, therefore, makes it possible to easily monitor a denaturation curve between a probe according to the present invention and a target nucleic acid. As the

difference in Tm value between Probe 100, which matches 100% with the target nucleic acid, and a probe, which mismatches by 1 base with the nucleic acid, is as much as 10℃ or greater, they can be easily identified from their denaturation curves. It is therefore understood that an analysis of SNPs can be conducted with ease by using a DNA chip according to the present invention.

PCR methods according to the present invention will hereinafter be described in Examples 24-27.

Example 24

Using as a target nucleic acid the 16S rRNA gene in the genome DNA of *Escherichia coli*, a primer labeled with "BODIPY FL/C6" (a nucleic acid probe according to the present invention) was prepared for the amplification of the nucleic acid.

Preparation of Primer 1 (Eu800R: reverse type)

An oligodeoxyribonucleotide having a base sequence of (5')CATCGTTTAC GGCGTGGAC(3') was synthesized using a DNA synthesizer, "ABI 394" (trade name; manufactured by Perkin-Elmer, Corp.). An oligonucleotide, which had been prepared by treating the phosphate group at the 5'end of the oligodeoxyribonucleotide with phosphatase to form cytosine and then bonding $-(CH_2)_9-NH_2$ to the OH group on the carbon atom at the 5'-position of the cytosine, was purchased from Midland Certified Reagent Company. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased,

which contained not only "BODIPY FL/C6" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above-purchased oligonucleotide, whereby Primer 1 of the present invention labeled with "BODIPY FL/C6" was synthesized.

Purification of synthesized product

The synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M $Na_2CO_3$/$NaHCO_3$ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby Primer 1 of the present invention was obtained with a yield of 50% as calculated relative to 2 mM of the starting oligonucleotide.

The above-described reversed phase chromatography was conducted under the following conditions:

Eluting solvent A: 0.05 N TEAA 5% $CH_3CN$

Eluting solvent B (for gradient elution): 0.05 N TEAA 40% $CH_3CN$

Column: CAPCEL PAK C18 (trade name), 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40℃

Detection: 254 nm

Example 25

Preparation of Primer 2 (Eu500R/forward: forward type)

Primer 2 composed of an oligodeoxyribonucleotide, which had a base sequence of (5')CCAGCAGCCG CGGTAATAC(3'), and a fluorescent dye ("BODIPY FL/C6") labeled to the 5'end of the oligodeoxyribonucleotide, was prepared with a yield of 50% in a similar manner as in Example 24.

Example 26

Using a test tube containing a liquid culture medium (5 mL; composition: NB, 0.08 g/100 mL) of sterilized nutrient broth (NB) (product of Difco Laboratories Ltd., Surrey, Great Britain), *Escherichia coli* JM109 was cultivated overnight at 37℃ under shaking. A 1.5-mL aliquot of the culture was centrifuged in a 1.5-mL centrifuge tube, whereby cells were obtained. From the cells, genome DNA was extracted using "DNeasy Tissue Kit" (trade name, product of QIAGENE GmbH, Hilden, Germany). The extraction was conducted following the protocol of the kit. As a result, a 17-ng/μL DNA solution was obtained.

Example 27

Using the genome DNA of the above *E. coli* strain, Primer 1 and/or Primer 2, PCR reactions were conducted by a method known per se in the art while using "LightCycler™ System" (trade name) marketed from Roche Diagnostics, Mannheim, Germany.

Operations were conducted following the manual of the system.

In the above system, PCR was conducted as specified in the manual except that Primer 1 and/or Primer 2 of the present invention were used in place of nucleic acid probes (two nucleic acid probes making use of the FRET phenomenon) and a general primer (a general primer not labeled with any fluorescent dye), both of which are listed in the manual).

PCR was conducted in a hybridization mixture of the following components:

| | |
|---|---|
| *E. coli* genome DNA solution | 3.5 μL |
| (final concentration: 0 to 6 ng/20 μL) | |
| (final copy number: 0 to 2.4 x $10^6$ copies) | |
| Primer solution | 0.8 μL |
| (final concentration: 0.08 μM) | |
| Taq solution | 10.0 μL |
| "MiliQ" purified water | 5.7 μL |
| Final whole volume | 20.0 μL |

Incidentally, the experiments were conducted by using the target nucleic acid, *E. coli* 16S rDNA, at the concentrations of the respective experiment groups shown in the brief description of FIG. 15 and also by using the primers in the combinations of Primer 1 and/or Primer 2 also shown in the brief description of FIG. 15.

The above Taq solution is a mixed solution of the following reagents:

| | |
|---|---|
| Taq solution | 96.0 μL |
| "MiliQ" purified water | 68.2 μL |
| Taq DNA polymerase solution | 24.0 μL |
| Taq start | 3.8 μL |

Incidentally, these Taq solution and Taq DNA polymerase solution were both included in the "DNA Master Hybridization Probe Kit" (trade name; product of Roche Diagnostics, Mannheim, Germany). Specifically, as the Taq DNA polymerase solution, the 10 x conc. solution (red cap) was used by diluting it tenfold. Further, Taq start is an antibody for the Taq DNA polymerase and is marketed by Clontech Laboratories, Inc., CA, U.S.A. Addition of Taq start to a reaction mixture can suppress activity of Taq DNA polymerase up to 70℃. This means that "hot-start" PCR can be performed.

The following reaction conditions were used.

| | |
|---|---|
| Denaturation | Initial: 95℃, 120 seconds |
| | Second and onwards: 95℃, 10 seconds |
| Annealing | 57℃, 5 seconds |

Measurements were conducted using "LightCycler™ System" (manufactured by Roche Diagnostics, Mannheim Germany). For each measurement, the detector F1 was used out of the detectors F1-F3 included in the system, and the gain and excitation level of the detector were set at 10 and 75, respectively.

The results are shown in FIG. 15 and FIG. 16. It is appreciated from FIG. 15 and FIG. 16 that the number of cycles

at the time of observation of a decrease in fluorescence emission from the fluorescent dye and the number of copies of *E. coli* 16S rDNA as the target nucleic acid are proportional to each other. In these diagrams, decreases in fluorescence emission from the fluorescent dye are expressed in terms of decreases in the intensity of fluorescence.

FIG. 17 shows a working line for *E. coli* 16S rDNA, in which the number of copies of *E. coli* 16S rDNA is expressed as a function of cycles. The correlation coefficient was 0.9973, so that an extremely good correlation was exhibited.

As is understood from the above results, use of the quantitative PCR method of the present invention makes it possible to count the initial number of copies of a target nucleic acid. This means that the concentration of the target nucleic acid can be determined.

Example 28

In Example 27, PCR was conducted using the invention probes as primers. In this example, however, PCR according to the present invention was conducted under the following conditions by using a primer of the present invention as opposed to two probes required in the conventional method making use of the FRET phenomenon.

a) Target nucleic acid: 16S rDNA of *Escherichia coli*

b) Primers:

- Forward primer E8F: (5')AGAGTTTGAT CCTGGCTCAG(3')

- Reverse primer E1492R:

(5′)GGTTACCTTG TTACGACTT(3′)

c) Probe: BODIPY FL-(5′)CGGGCGGTGT GTAC(3′) (with the 3′end phosphorylated)

d) PCR apparatus: "LightCycler™ System" (trade mark, manufactured by Roche Diagnostics GmbH, Mannheim, Germany)

e) Conditions for PCR:

Denaturation: 95℃ for 10 seconds (95℃ for 60 seconds in the first cycle only)

Annealing: 50℃ for 5 seconds

Extension: 72℃ for 70 seconds

Total cycle number: 70 cycles

f) Fluorescence assay (measurement):

Assay (measurement) was performed once after each of denaturation and annealing in each cycle.

g) Composition of reaction mixture:

Total volume: 20 μL

Amount of DNA polymerase ("TaKaRa Ex taq"): 0.5 U

Amount of TaqStart (antibody): 0.3 μL

Concentration of primer: 0.2 μM (common to both primers)

Concentration of probe: 0.05 μM

Concentration of $MgCl_2$: 2 mM

Conc. of BSA (bovine serum albumin): 0.25 mg/mL

Concentration of dNTPs: 0.2 mM (for each nucleotide)

The results are shown in FIG. 18. It is understood from the diagram that the number of cycles at the time of observation of a decrease in fluorescence emission from the fluorescent dye and the number of copies of *E. coli* 16S rDNA as the target nucleic acid are proportional to each other.

As is understood from the above results, use of the quantitative PCR method of the present invention makes it possible to count the initial number of copies of a target nucleic acid. This means that the concentration of the target nucleic acid can be determined.

In the subsequent Examples, the data analysis method of the present invention for analyzing data obtained by using the above-described quantitative PCR method of the present invention will be described.

Example 29

Using, as a target nucleic acid, human genome DNA (human β-globin) (TaKara Catalog Product No. 9060) (product of TAKARA SHUZO CO., LTD., Kyoto Japan) (hereinafter called "the human genome DNA"), a primer labeled with "BODIPY FL/C6" was prepared for the amplification of the nucleic acid.

Preparation of Primer KM38+C (reverse type)

An oligodeoxyribonucleotide having a base sequence of (5')CTGGTCTCCT TAAACCTGTC TTG(3') was synthesized using a DNA

synthesizer, "ABI 394" (trade name; manufactured by Perkin-Elmer, Corp.). An oligonucleotide, which had been prepared by treating the phosphate group at the 5'end of the oligodeoxyribonucleotide with phosphatase to form cytosine and then bonding $-(CH_2)_9-NH_2$ to the OH group on the carbon atom at the 5'-position of the cytosine, was purchased from Midland Certified Reagent Company. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL/C6" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above-purchased oligonucleotide, whereby Primer KM38+C of the present invention labeled with "BODIPY FL/C6" was synthesized.

Purification of synthesized product

The synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby Primer KM38+C of the present invention was obtained with a yield of 50% as calculated relative to 2 mM of the starting

oligonucleotide.

The above-described reversed phase chromatography was conducted under the following conditions:

Eluting solvent A: 0.05 N TEAA 5% $CH_3CN$

Eluting solvent B (for gradient elution): 0.05 N TEAA 40% $CH_3CN$

Column: "CAPCEL PAK C18" (trade name), 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40℃

Detection: 254 nm

Example 30

Preparation of Primer KM29 (forward type)

An oligodeoxyribonucleotide having a base sequence of (5')GGTTGGCCAA TCTACTCCCA GG(3') was synthesized in a similar manner as in Example 26.

Comparative Example 1

This Comparative Example is directed to use of a data analysis software which did not include the processing step that an intensity of fluorescence during an extending reaction of a nucleic acid is divided using an intensity of fluorescence at the time of a thermal denaturing reaction [i.e., the processing of the formula (1)].

Using the above-described human genome DNA, Primer KM38+C and Primer KM29, PCR reactions were conducted by "LightCycler™ System". The intensity of fluorescence was measured in each

cycle.

Incidentally, the PCR in this Comparative Example employed the above-described primers labeled with the fluorescent dye, and is a novel real-time quantitative PCR method in which a decrease in fluorescence emission is measured rather than an increase in fluorescence emission. An analysis of data was conducted using the software of the system itself. The PCR in this Comparative Example was conducted following the manual of the system except that the invention primers KM38+C and KM29 were used instead of the nucleic acid probes listed in the manual (two probes making use of the FRET phenomenon) or an ordinary primer (an ordinary primer not labeled with any fluorescent dye).

PCR was conducted in a hybridization mixture of the following components:

| | |
|---|---|
| Human genome DNA | 1.0 μL |
| (final concentration: 1 to 10,000 copies) | |
| Primer solution | 4.0 μL |
| (final concentration: 0.1 μM) | |
| Taq solution | 10.0 μL |
| "MiliQ" purified water | 5.0 μL |
| Final whole volume | 20.0 μL |

Incidentally, the experiments were conducted by using the human genome DNA at the concentrations of the respective experiment groups shown in the brief description of FIG. 19.

The final concentration of $MgCl_2$ was 2 mM.

The above-described "Taq solution" is a liquid mixture of the following reagents:

| | |
|---|---|
| Taq solution | 96.0 μL |
| "MiliQ" purified water | 68.2 μL |
| Taq DNA polymerase | 24.0 μL |
| Taq start | 3.8 μL |

Incidentally, the "Taq solution" and the "Taq DNA polymerase solution" are included in "DNA Master Hybridization Probes" (trade name) marketed by Roche Diagnostic GmbH, Mannheim, Germany. Specifically, the "Taq DNA polymerase solution" was used by diluting "10 x conc." (red cap) tenfold. Further, the "Taq start" is an antibody to Taq DNA polymerase, and is marketed by Clontech laboratories, Inc., CA, U.S.A. Its addition to the reaction mixture makes it possible to inhibit the activity of Taq DNA polymerase up to 70℃. In other words, "hot-start" PCR can be performed.

The following reaction conditions were used.

| | |
|---|---|
| Denaturation | Initial: 95℃, 60 seconds |
| | Second and onwards: 95℃, 10 seconds |
| Annealing | 60℃, 5 seconds |

DNA extending reaction: 72℃, 17 seconds

Measurements were conducted using "LightCycler™ System". For each measurement, the detector F1 was used out of the detectors F1-F3 included in the system, and the gain and

excitation level of the detector were set at 10 and 75, respectively.

PCR was conducted as described above, during which the intensities of fluorescence in individual cycles were measured. The results are shown in FIG. 19. Described specifically, with respect to each of the human genome DNAs of the respective copy numbers, the intensity of fluorescence was measured at the time of a denaturing reaction and also at the time of a nucleic acid extending reaction, both in each cycle, and was printed. It is observed that the intensity of fluorescence remained constant at the time of the denaturing reaction irrespective of the cycle but a decrease in fluorescence took place from the 25th cycle at the time of the nucleic acid extending reaction. It is also understood that this decrease occurs earlier as the number of copies of the human genome DNA increases.

As is shown in FIG. 19, the intensities of fluorescence in initial cycles were not constant irrespective of the number of copies of the human genome DNA. The following steps (b)-(j) were, therefore, added to the data analysis method for use in this Comparative Example.

(b) Assuming that the intensity of fluorescence in the $10^{th}$ cycle is 1, the intensity of fluorescence in each cycle is converted, namely, calculation is conducted in accordance with the following formula (8):

$$C_n = F_n(72)/F_{10}(72) \qquad (8)$$

where

$C_n$: a converted value of the intensity of fluorescence in each cycle,

$F_n(72)$: the intensity of fluorescence at 72℃ in each cycle, and

$F_{10}(72)$: the intensity of fluorescence after extending reaction at 72℃ in the $10^{th}$ cycle.

(c) Each converted value obtained in step (b) is displayed on a display and/or printed as a function of cycle.

(d) From the converted value in each cycle as obtained in step (b), the rate of a change in fluorescence intensity (decrease or quench, %) is calculated in accordance with the following formula (9):

$$F_{dn} = \log_{10}\{100-C_n \times 100)\} \quad (9)$$

$$F_{dn} = 2\log_{10}\{1-C_n\} \quad (9)$$

where

$F_{dn}$: the rate of a change in fluorescence intensity (decrease or quench, %), and

$C_n$: the value obtained in accordance with the formula (8).

(e) Each converted value obtained in step (d) is displayed on a display and/or printed as a function of cycle.

(f) Data processed in step (d) are compared with 0.5 as a threshold, and the number of cycles the data of which reach the threshold is counted.

(g) A graph is prepared by plotting values, which have been counted in step (f), along X-axis and the numbers of copies before the initiation of the reaction along Y-axis.

(h) The graph prepared in step (g) is displayed on a display and/or printed.

(i) A correlation coefficient or relational formula of the line drawn in step (h) is calculated.

(j) The correlation coefficient or relational formula calculated in step (i) is displayed on a display and/or printed.

Using the above-described data analysis software, the data obtained in FIG. 19 were then processed as will be described hereinafter.

FIG. 20 is a print-out of the data processed in step (b) [process (c)]. Namely, assuming that the intensity of fluorescence in the $10^{th}$ cycle was 1, the fluorescence intensities in the individual cycles were converted, and the converted values were plotted against the corresponding cycles.

FIG. 21 is a print-out of the data processed in step (d) [process (e)]. Namely, decreases (%) (quenches, %) of the respective fluorescence intensities were calculated from the plotted values in FIG. 20, and the values so calculated were plotted against the corresponding cycles.

FIG. 22 is a print-out of the graph prepared in step (g) based on the data processed in step (f) [step (h)]. Namely, it is a graph obtained by using a decrease of 0.5 in fluorescence

intensity as a threshold, plotting along X-axis the number of cycles in which the threshold was reached, and also plotting along Y-axis the numbers of copies of the human genome DNA before the initiation of the respective reactions. The correlation coefficient (R2) of the line in this graph was calculated in step (i), and was then printed [step (j)]. The correlation coefficient was 0.9514. As is understood, it was hardly possible, with this correlation coefficient, to determine an accurate number of copies this correlation coefficient was

Example 31

(This Example is directed to an experiment in which processing of data was performed by using the data analysis method of the present invention)

PCR was conducted in a similar manner as in Comparative Example 1. The processing of the data was performed through similar steps as in Comparative Example 1 except that the following step (a) was added before the step (b) and the steps (b),(d) were modified as will be described below.

(a) The intensity of fluorescence in each cycle in a reaction system in which an amplified nucleic acid hybridized to a nucleic acid primer labeled with a fluorescent dye as a nucleic acid probe of the present invention [namely, the intensity of fluorescence at the time of a nucleic acid extending reaction (72℃)] was corrected in a correction processing step such that the intensity of fluorescence was divided by the intensity of

fluorescence in the reaction system measured at the time of dissociation of the nucleic acid hybrid complex (the hybrid complex formed by hybridization of the amplified nucleic acid and the nucleic acid primer [namely, the intensity of fluorescence at the time of completion of the thermal denaturing reaction of the nucleic acid (95℃)], that is, the actually-measured intensities of fluorescence were corrected in accordance with the following formula (1):

$$f_n = f_{hyb,n}/f_{den,n} \qquad (1)$$

where

$f_n$: a correction value for the intensity of fluorescence in each cycle,

$f_{hyb,n}$: the intensity of fluorescence at 72℃ in each cycle, and

$f_{den,n}$: the intensity of fluorescence at 95℃ in each cycle.

It is FIG. 23 that was obtained by plotting the thus-obtained values against the corresponding cycles.

(b) A processing step that the values correction-processed by formula (1) in the respective cycles were introduced into the formula (3) to calculate the rates of changes (decreases or quenches, %) in fluorescence between the samples in the respective cycles, namely, a step for performing processing in accordance with the following formula (10):

$$F_n = f_n/f_{25} \qquad (10)$$

where

$F_n$: a processed value in each cycle,

$f_n$: a value of each cycle as obtained in accordance with formula (1), and

$f_{25}$: a value of the $25^{th}$ cycle as obtained in accordance with formula (1).

Formula (10) is similar to formula (3) except for a=25.

(d) A step that the processed value of each cycle as obtained in step (b) was subjected to processing in accordance with formula (6) to obtain the logarithm of the rate of a change (decrease or quench, %) in fluorescence intensity, namely, a step for performing processing in accordance with the following formula (11):

$$\log_{10}\{(1-F_n) \times 100\} \qquad (11)$$

where

$F_n$: value obtained in accordance with formula (10).

Formula (11) is similar to formula (6) except for b=10 and A=100.

The above results are shown in FIGS. 24 and 25.

FIG. 24 is a print-out obtained by plotting the values, which have been processed in steps (a) and (b), against the corresponding cycles.

FIG. 25 is a print-out obtained by processing the values, which have been obtained in FIG. 24, in a similar manner as in step (d) and then plotting the thus processed values against

the corresponding cycles.

Next, based on the graph of FIG. 25, processing was performed through steps (f),(g) and (h). Described specifically, as in Comparative Example 1, 0.1, 0.3, 0.5, 0.7, 0.9 and 1.2 were chosen as thresholds for $\log_{10}$ (rates of changes in fluorescence intensity, %) on the basis of the graph of FIG. 25. The numbers of cycles in which the logarithms reached the thresholds were plotted along X-axis, while the numbers of copies of the human genome DNA before the initiation of reactions were plotted along Y-axis, whereby working lines were drawn. The results are shown in FIG. 26. Correlation coefficients ($R^2$) determined by conducting processing in steps (i) and (j) with respect to those working lines were 0.998, 0.999, 0.9993, 0.9985, 0.9989 and 0.9988, respectively. From those correlation coefficients, it was able to confirm that adoption of 0.5 as a threshold (correlation coefficient: 0.9993) is desired. It is understood that, with a working line having this correlation coefficient, the number of copies before initiation of a reaction can be accurately determined with respect to a nucleic acid sample the number of copies of which is unknown.

Example 32

This Example is directed to an analysis of a melting curve of a nucleic acid and also to an analysis of a Tm value.

A software comprising the following steps was created:

1) with respect to a nucleic acid amplified by the novel PCT

method of the present invention, gradually heating the amplified nucleic acid from a low temperature until the nucleic acid is completely denatured (for example, from 50℃ to 95℃), or gradually lowering it; 2) in step 1), measuring the intensity of fluorescence at short time intervals (for example, at intervals equivalent to temperature rises of from 0.2℃ to 0.5℃); 3) displaying the measurement results of step 2) on a display as a function of time, namely, displaying a melting curve of the nucleic acid; 4) differentiating the melting curve obtained in step 3); 5) displaying, on a display, derivatives (-dF/dT, F: fluorescence intensity, T: time) obtained in step 4); and 6) determining a point of inflection from the derivatives obtained in step 5). The software was combined with the above-described data analysis software of the present invention. Using "LightCycler™ System" in which a computer-readable recording medium with the data analysis software recorded therein had been installed, the novel real-time quantitative PCR reaction of the present invention was conducted to analyze the melting curve of the nucleic acid. In the present invention, the intensity of fluorescence increases with the temperature.

With respect to 1 copy and 10 copies of the same human genome DNA as in Example 31, PCR was conducted in a similar manner as in Example 29. FIG. 27 is a print-out of data obtained by processing data of the PCR in steps 1), 2), 3), 4) and 5).

Concerning 75$^{th}$ amplification products of the 1 copy and 10 copies, data were processed in steps 1), 2 and 3) of this Example. The nucleic acid melting curves so obtained are shown in FIG. 28. Those curves were differentiated in step 4), and points of inflection (Tm values) were determined in steps 5) and 6). The differentiated curves with the points of inflection are illustrated in FIG. 29. It was ascertained from FIG. 29 that the amplification products of the 1 copy and 10 copies were different products as their Tm values were different from each other.

The following Examples relate to quantitative polymorphous analysis methods.

Example 33

(Preparation of fluorescence quenching probes according to the present invention, Probe Eu47F and Eu1392R)

(1) Synthesis of the fluorescence quenching probe Eu47F

The fluorescence quenching probe Eu47F, which was composed of a deoxyribooligonucleotide having the base sequence of (5')CITAACACATGCAAGTCG(3')(I: inosine) and labeled on the phosphate group at the 5'end thereof with "BODIPY FL" as will be described below, was synthesized by a DNA synthesizer "ABI 394" (trade name, manufactured by Perkin-Elmer Inc., MA, U.S.A.).

(2) Synthesis of Eu1392R

A deoxyribooligonucleotide the base sequence of which was

(5')TTGTACACACCGCCCGTCA(3') was synthesized.

The deoxyribooligonucleotide with $-(CH_2)_6-NH_2$ bound on the phosphate group at the 5'end thereof was purchased from Midland Certified Reagent Company, TX, U.S.A. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above-purchased oligonucleotide to synthesize the above-described invention fluorescence quenching probe labeled with "BODIPY FL".

Incidentally, purification of each of the above-described synthesized products was conducted as will be described hereinafter.

Each synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby the target product was obtained with a yield of 50% as calculated relative to 2 mM of the starting oligonucleotide.

Conditions for reversed phase chromatography:

Eluting solvent A: 0.05 N TEAA 5% $CH_3CN$

Eluting solvent B (for gradient elution): 0.05 N TEAA 40% $CH_3CN$

Column: "CAPCEL PAK C18" (trade name), 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40℃

Detection: 254 nm

Example 34

(1) Cultivation of *Escherichia coli* JM109

Using Medium 53 (composition: casein peptone (tripsin digest of casein), 10 g; yeast extract, 5 g; glucose, 5 g; salt, 5 g; distilled water, 1000 mL), *Escherichia coli* JM109 was cultivated (culture medium 50 mL/250 mL Erlenmeyer flask, 37℃, 12 hours, shaking culture). Cells were collected from the culture (centrifugation under 10,000 rpm for 5 minutes, washed twice with distilled water).

(2) Preparation of DNA of 16S rRNA

Using "SOGEN Kit" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan), whole RNAs were extracted from the cells in accordance with the protocol of the kit.

Using "BcaBEST™ RNA PCR Kit" (product of Takara Shuzo Co., Ltd., Kyoto, Japan), the extract was subjected with respect to 16s RNA to amplification and reverse transcription reaction (RT-PCR) under known usual conditions in accordance with the

protocol of the kit. Upon these amplification and reverse transcription reaction (RT-PCR), the above-described fluorescence quenching probe EU1392R according to the present invention was used as a primer. Subsequently, RNA was cleaved by Rnase H (30℃, 20 minutes), and pure cDNA of the 16S rRNA gene was obtained. The concentration of cDNA was determined using "OliGreen$^{R}$ssDNA Quantitation Kit" (trade name; product of Molecular Probes, Inc., OR, U.S.A.).

Example 35

(1) Quantitative PCR, data analysis, and preparation of working curves for cDNA

With respect to the above-described cDNA solution, a real-time monitoring quantitative PCR reaction was conducted using the invention fluorescence quenching probe EU47F as a forward primer and the invention fluorescence quenching probe Eu1392R as a reverse primer.

Using "LightCycler™ System" (trade name, manufactured by Roche Diagnostic GmbH, Mannheim, Germany) as a real-time monitoring quantitative PCR system, a reaction was conducted in accordance with the procedures described in the manual. Incidentally, "TaKaRaTaq™" (product of Takara Shuzo Co., Ltd., Kyoto, Japan) was used as DNA polymerase.

PCR was conducted with the following components:

*E. coli* cDNA 3.5 μL

(final concentration: $10^2$ to copies)

| | |
|---|---|
| Primer solution | 4.0 μL |
| (final concentration: 0.1 μM) | |
| TaKaRaTaq™ | 10.0 μL (0.5 unit) |
| "MiliQ" purified water | 5.0 μL |
| Final whole volume | 20.0 μL |

Incidentally, the experiment was conducted using the cDNA in the copy numbers specified in the brief description of FIG. 30. The final concentration of $MgCl_2$ was 2 mM.

The reaction was conducted under the following conditions:

| | |
|---|---|
| Denaturation | Initial: 95℃, 60 seconds |
| | Second and onwards: 96℃, 10 seconds |
| Annealing | 50℃, 5 seconds |
| DNA extension: | 72℃, 60 seconds |

Measuring conditions were set as follows:

| | |
|---|---|
| Exciting light: | 488 nm |
| Measuring fluorescent color: | 530 nm |

Real-time monitoring quantitative PCR was conducted under similar conditions as described above, and the intensities of fluorescence in individual cycles was actually measured. The actually measured values were analyzed in accordance with the data analysis method of the present invention. Specifically, the data were processed through the following steps:

(a) The intensity of fluorescence in each cycle in the

reaction system in which the amplified nucleic acid hybridized to the nucleic acid primer labeled with the fluorescent dye [namely, the intensity of fluorescence at the time of the nucleic acid extending reaction (72℃)] was corrected in a correction processing step such that the intensity of fluorescence was divided by the intensity of fluorescence in the reaction system measured at the time of complete dissociation of the nucleic acid hybrid complex (the hybrid complex formed by hybridization of the amplified nucleic acid and the nucleic acid primer [namely, the intensity of fluorescence at the time of completion of the thermal denaturing reaction of the nucleic acid (96℃)], that is, the actually-measured intensities of fluorescence were corrected in accordance with the following formula (1):

$$f_n = f_{hyb,n}/f_{den,n} \qquad (1)$$

where

$f_n$: a correction value for the intensity of fluorescence in each cycle,

$f_{hyb,n}$: the intensity of fluorescence at 72℃ in each cycle, and

$f_{den,n}$: the intensity of fluorescence at 96℃ in each cycle.

(b) A processing step that the values correction-processed by formula (1) in the respective cycles were introduced into the formula (3) to calculate the rates of quenches (%) in

fluorescence between the samples in the respective cycles, namely, a step for performing processing in accordance with the following formula (10):

$$F_n = f_n/f_{25} \qquad (10)$$

where

$F_n$: a processed value in each cycle,

$f_n$: a value of each cycle as obtained in accordance with formula (1), and

$f_{25}$: a value of the $25^{th}$ cycle as obtained in accordance with formula (1).

Formula (10) is similar to formula (3) except for a=25.

(c) A step that the processed value of each cycle as obtained in step (b) was subjected to processing in accordance with formula (6) to obtain the logarithm of the rate of a change (decrease or quench, %) in fluorescence intensity, namely, a step for performing processing in accordance with the following formula (11):

$$\log_{10}\{(1-F_n) \times 100\} \qquad (11)$$

where

$F_n$: value obtained in accordance with formula (10).

Formula (11) is similar to formula (6) except for b=10 and A=100.

The above results are shown in FIG. 30.

FIG. 30 is a print-out obtained by plotting the values, which have been calculated in steps (a), (b) and (c), against

the corresponding cycles.

Next, based on the graph of FIG. 30, processing was performed through the following steps (d) and (e).

(d) A step that data processed in step (c) are compared with 0.2 as a threshold, and the number of cycles the data of which reach the threshold is counted.

(e) A step that a graph is prepared by plotting values, which have been calculated in step (d), along X-axis and the numbers of copies before the initiation of the reaction along Y-axis, that is, a working line (FIG. 31) for *Escherichia coli* cDNA is prepared.

FIG. 31 shows the final results obtained when data obtained by the quantitative PCR method of the present invention were processed by the data analysis method of the present invention, namely, through steps (a), (b), (c), (d) and (e).

Example 36

(1) Construction of a polymorphous system (a co-cultivation system of microorganisms)

Ten (10) bacteria strains shown in Table 5 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Using Medium 53 described above, they were separately cultured. Culture conditions were similar to the above-described conditions for *Escherichia coli*. From each culture, cells were collected (centrifugal separation at 10,000 rpm for 10 minutes; washed

twice with distilled water). From each sample of cells, whole RNAs were extracted in a similar manner as described above by using "SOGEN Kit" (trade name; product of NIPPON GENE CO., LTD., Tokyo, Japan).

Table 8

| Strain No. | | DSMZ No. | HhaI fragment (bp) | Molar fraction (%) determined from T-RFLP | Number of quantitated copies | Number of quantitated copies/number of initially added copies |
|---|---|---|---|---|---|---|
| 1 | *Paracoccus pantotrophus* | 65 | 22 | 9.5 | 27400 | 0.91 |
| 2 | *Sphingomonas natatoria* | 3138T | 43 | 10.9 | 31400 | 1.05 |
| 3 | *Bdellovibrio stolpii* | 12778 | 52 | 9.7 | 27900 | 0.93 |
| 4 | *Microbacterium imperiale* | 20530 | 104 | 9.4 | 27100 | 0.90 |
| 5 | *Pseudomonas fluorescens* | 50108 | 168 | 10.4 | 30000 | 1.00 |
| 6 | *Agromyces medislanum* | 20152 | 332 | 9.3 | 26800 | 0.89 |
| 7 | *Cellulomonas cellulans* | 43879 | 404 | 9.7 | 27900 | 0.93 |
| 8 | *Brevibacterium liquefaciens* | 20579 | 432 | 9.9 | 28500 | 0.95 |
| 9 | *Leminorella grimontii* | 5078 | 531 | 10.4 | 30000 | 1.00 |
| 10 | *Rhodococcus luteus* | 43673 | 626 | 10.8 | 31100 | 1.04 |

In a similar manner as in the above-described case of *Escherichia coli*, pure cDNAs of the 16S rRNA genes of the respective strains were obtained. The respective concentrations of the thus-obtained cDNAs of the 10 strains were determined in a similar manner as in the above-described case of *Escherichia coli*. The solutions the cDNA concentrations of which had been ascertained were diluted with distilled water to 300,000 copies/μL. Concerning the 10 strains, the diluted solutions were mixed in equal amounts to provide a co-cultivation system of microorganisms, in other words, a polymorphous system (hereinafter called a "polymorphous system"). As the cDNAs of the 10 strains are each contained at the concentration of 300,000 copies/μL in the polymorphous system, the cDNAs are contained as a whole at a concentration of 3,000,000 copies/μL.

(2) Real-time monitoring quantitative PCR

With respect to the cDNAs in the above-described polymorphous system, real-time monitoring quantitative PCR was conducted in a similar manner as in the above-described *Escherichia coli* by using the fluorescent quenching probes Eu47F and Eu1392R of the present invention as primers common to the strains.

A polymorphous sample was added to a reaction mixture to give a concentration of 300,000 copies/20 μL in terms of absolute count. In real-time monitoring quantitative PCR of

the polymorphous system, the reaction was terminated in the 22nd cycle in which a decrease in the intensity of fluorescence was observed and which was an exponential growth phase of the genes (see FIG. 30). The number of copies of cDNAs in the reaction mixture of real-time monitoring quantitative PCR conducted on the polymorphous system was 288,000 copies (see FIG. 31). Since the initially-added amount, that is, the calculated count was 300,000 copies, the working line prepared by the method of the present invention has been confirmed to show good quantitativeness.

Example 37

(Polymorphous analysis)

(1) Analysis by T-RFLP

After a PCR reaction was conducted as described above, amplified products were purified using a column ("Microcon PCR", trade name; product of Millipore Corporation, Bedford, MA, U.S.A.). Purified products were treated overnight with a restriction endonuclease HhaI (recognition site: GCG/C, /: cleaved site). After completion of the treatment, only cleaved fragments were purified through columns ("Microcon" and "Micropure-Ez", trade names; products of Millipore Corporation, Bedford, MA, U.S.A.). The sizes of cDNA fragments of the respective strains after the treatment with the restriction endonuclease are shown in Table 8.

The cDNA solution, to which the column chromatographic

purification had been applied, was subjected to thermal denaturation, followed by a T-RFLP analysis by a sequencer ("ABI PRISMTH 310", trade name; manufactured by Perkin Elmer - Applied Biosystems Inc., CA, U.S.A.). A peak pattern of the T-RFLP analysis is shown in FIG. 32. Each peak was quantitated using a standard "BODIPY FL"-modified fragment the concentration of which was known. The molar fractions (%) of the individual peaks were determined. As a result, the molar fractions (%) all fell within a range of from 9.4 to 10.8 and no substantial difference was observed in the efficiencies of PCR amplification of the cDNA fragments of the respective strains (see Table 8). The ratio of the number of quantitated copies to the number of initially added copies ranged from 0.89 to 1.04 (see Table 8). It has hence been found that the numbers of initial copies of cDNAs of the individual strains in a polymorphous system can be accurately quantitated by this method.

Example 38

[Example directed to a real-time quantitative PCR method making use of fluorescence emitting probes as primer (hereinafter called "fluorescence emitting primers") and a quantitative polymorphous analysis method making use of the real-time quantitative PCR method]

A description will be made about an Example directed to a real-time quantitative PCR method making use of fluorescence

emitting probes and a quantitative polymorphous analysis method making use of the real-time quantitative PCR method.

1) Experimental procedures and conditions

<Preparation of an artificial co-cultivation system of microorganisms (template)>

An artificial co-cultivation system of microorganisms was prepared. Using it as a model system, effectiveness of a quantitative polymorphous analysis method was proven. For the experiment, 10 kinds of microorganisms shown in Table 9 were purchased from DSMZ. The individual strains were separately cultivated using Medium 53. From the cultures, cells were collected, and total DNAs were extracted with a kit reagent "ISOGEN" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan) in accordance with its protocol. Using Eu47F (CITAACACATGCAAGTCG, I: inosine) and Eu1392R (TTGTACACACCGCCCGTCA) as primers, a PCR reaction was conducted on 16s RNA genes as amplification targets. The thus-amplified products of the 10 kinds of 16S rRNA genes were quantitated by "PicoGreen®dsDNA Quantitation Kit" (trade name, product of Molecular Probes, Inc., OR, U.S.A.), and were then separately diluted with sterilized distilled water to give a concentration of 300,000 copies/mL. The thus-diluted solutions were mixed in equal amounts to provide an artificial co-cultivation system model of microorganisms. This artificial co-cultivation system model of microorganisms contained amplified products of

16S rRNA genes of the 10 microorganisms at concentrations of 30,000 copies/mL, respectively. The total concentration of the amplified products of the 16S rRNA genes was, therefore, 300,000 copies/mL.

<Procedures of a real-time quantitative PCR experiment making use of fluorescence emitting primers according to the present invention>

Using the above-described artificial co-cultivation system of microorganisms (the mixed 16S rRNA gene sample) as a target, quantitative PCR was conducted using fluorescence emitting primers dually modified with Texas Red and Dabcyl. Employed as common primers were Eu47F-modi (CITAACACATGCAAGTCG, I: inosine) and Eu1392R (TTGTACACACCGCCCGTCA). Eu47F-modi had a similar base sequence as Eu47F, but the 9$^{th}$ T from the 5'end was modified with Texas Red and the 9$^{th}$ T was modified with Dabcyl. The modifications with Texas Red and Dabcyl were conducted in a similar manner as in Example 7. As a quantitative PCR apparatus, "iCycler" (trade mark, manufactured by Bio-Rad Laboratories, Inc., CA, U.S.A.) was used. The first denature was carried out at 95℃ for 60 seconds, and PCR cycles were conducted under the following conditions: denature: 95℃/60 seconds, annealing: 50℃/60 seconds and extension: 72℃/70 seconds. The PCR reaction was terminated in an exponential growth phase such that the initial composition of the genes would not be altered (no PCR bias would be applied). As the

concentrations of the primers, Eu47F and Eu1932R were both set at 0.1 μM, respectively, in terms of final concentration. As a DNA polymerase, "TaKaRa TaqTM" (trade name, product of Takara Shuzo Co., Ltd., Kyoto, Japan) was used at a concentration of 0.5 unit/20 μL. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively. Using "AntiTaq body" (trade name, product of Clontech Laboratories, Inc., CA, U.S.A.), "hot-start" PCR was conducted following the maker's instruction manual. As a standard sample for the preparation of a working line, an amplified product of the 16S rDNA gene of *E. coli* was used. The preparation of the amplified product of the 16S rDNA gene of *E. coli* was conducted in a similar manner as the above-described artificial co-cultivation system of microorganisms.

Subsequent to the preparation of the working line, quantitation of the artificial co-cultivation system of microorganisms was conducted. The gene concentration of the artificial co-cultivation system of microorganisms was adjusted to give a concentration of 300,000 copies/20 μL in terms of absolute count (20 μL: total amount). Measurement of fluorescence was conducted once after denature and once after annealing in each cycle. Similarly to the quenching rate of fluorescence (%), the emitting rate of fluorescence (%) was determined by correcting the intensity of fluorescence after annealing (at the time of hybridization) with the intensity of fluorescence

after denaturation (at the time of dissociation).

A specific calculation formula can be expressed as:

$$F_n = \{(f_{hyb'n}/f_{den'n})/(f_{hyb'n'}/f_{den'n'})\} \times 100$$

where

$F_n$: Emitting rate of fluorescence (%) in the $n^{th}$ cycle,

$f_{hyb'n}$: Intensity of fluorescence during annealing (hybridization) in the $n^{th}$ cycle,

$f_{den'n}$: Intensity of fluorescence during denaturation (dissociation) in the $n^{th}$ cycle,

$f_{hyb'n'}$: Intensity of fluorescence after annealing (hybridization) in a cycle ($n'^{th}$ cycle) preceding occurrence of an emission of fluorescence from an amplified product, and

$f_{den'n'}$: Intensity of fluorescence after denaturation (dissociation) in the cycle ($n'^{th}$ cycle) preceding the occurrence of the emission of fluorescence from the amplified product.

<Analysis by T-RFLP>

After completion of the real-time quantitative PCR reaction, purification of the amplified products was conducted through a column ("Microcon PCR", trade name; product of Millipore Corporation, Bedford, MA, U.S.A.). Purified products were treated overnight with a restriction endonuclease HhaI (recognition site: GCG/C, /: cleaved site). After completion of thermal denaturation, a solution which contained

restriction fragments was subjected to a T-RFLP analysis by a sequencer ("ABI PRISMTH 310", trade name; manufactured by Perkin Elmer - Applied Biosystems Inc., CA, U.S.A.). After the individual restriction fragments were quantitated using fluorescence emitting probes of the same chain lengths as standards, respectively, the molar fractions (%) of the individual peaks were determined.

2) Results

<Results of real-time quantitative PCR making use of fluorescence emitting primers>

The results are shown in FIG. 33 and FIG. 34. As is appreciated from FIG. 33, it has been confirmed that monitoring of amplified products is feasible by using fluorescence emitting primers. Further, a relationship between the number of cycles required to reach a threshold [log $F_n$ (emitting rate of fluorescence, %) = 1.6] and the count of DNA copies added initially is illustrated in FIG. 34. As is readily appreciated from this diagram, it is understood that the number of cycles and the number of copies added initially is in a linear relationship. Accordingly, it is indicated from this diagram that the quantitation of initial copies of a target gene can be accurately achieved from the $n^{th}$ number of a cycle in which the threshold is reached. In the artificial co-cultivation system of microorganisms, the PCR reaction was terminated in a cycle ($23^{rd}$ cycle)in which logarithmic growth was observed

(see FIG. 33). From the working line shown in FIG. 34, the number of copies of the 16S rRNA in the artificial co-cultivation system of microorganisms was quantitated to be about 296,000 copies. Since the count of the initially added copies was 300,000 copies, the good quantitativeness of this method was confirmed.

<Results of the analysis by T-RFLP>

The amplified products of the real-time quantitative PCR were analyzed by the T-RFLP method to quantitate restriction fragments of the 16S rRNAs genes of the respective strains. As a result, the molar fractions (%) of all the peaks fell within a range of from 9.5 to 10.6, and no difference was observed in the efficiency of PCR amplification depending on the kind of 16S rNA gene (see Table 9). The number of initial copies of 16S rRNA gene of each constituent microorganism was determined by multiplying the total number of copies of 16S rRNA gene, which had been determined by the quantitative PCR, with the corresponding molar fraction (see Table 9). Concerning the 16S rRNA gene of each strain, the ratio of the number of quantitated copies to the number of initially added copies fell in a range of from 0.94 to 1.05 (see Table 9). It has hence been proven that the quantitation of initial copies of mixed genes in a artificial co-cultivation system of microorganisms (quantitation of target nucleic acids) by this method has good accuracy.

Table 9

Results of T-RFLP (Determined by Fluorescence Emitting Probes)

| Strain No. | DSMZ No. | Length of HhaI fragment (bp) | Molar fraction (%) determined from T-RFLP | Number of quantitated copies | Number of quantitated copies/number of initially added copies |
|---|---|---|---|---|---|
| *Paracoccus pantotrophus* | 65 | 22 | 9.50 | 28120 | 0.94 |
| *Sphingomonas natatoria* | 3183T | 43 | 10.10 | 29896 | 1.00 |
| *Bdellovibrio stolpii* | 12778 | 52 | 9.90 | 29304 | 0.98 |
| *Microbacterium imperiale* | 20530 | 104 | 9.60 | 28416 | 0.95 |
| *Pseudomonas fluorescens* | 50108 | 168 | 9.70 | 28712 | 0.96 |
| *Agromyces medislanum* | 20152 | 332 | 10.10 | 29896 | 1.00 |
| *Cellulomonas cellulans* | 43879 | 404 | 9.80 | 29008 | 0.97 |
| *Brevibacterium liquefaciens* | 20579 | 432 | 10.40 | 30784 | 1.03 |
| *Leminorella grimontii* | 5078 | 531 | 10.30 | 30488 | 1.02 |
| *Rhodococcus luteus* | 43673 | 626 | 10.60 | 31376 | 1.05 |

Example 39

(Example directed to a real-time quantitative PCR method making use of fluorescence emitting probes)

A description will be made about an Example of a real-time quantitative PCR method, the basic principle of which is to conduct quantitative PCR by using both prior art primers and fluorescence emitting probes according to the present invention and to conduct real-time monitoring of amplified products by the probes.

1) Experimental procedures and conditions

<Preparation of template DNA>

After the genome DNA of *Paracoccus denitrificans* DSM 413 was extracted by using "DNeasy™ Tissue Kit" (trade name, product of QIAGEN GmbH, Hilden, Germany), the 16S rRNA gene was amplified by conventional PCR while using a primer set consisting of E10F (AGAGTTTGATCCTGGCTCAG: not modified with any fluorescent dye) and E1400R(GGTTACCTTGTTACGACTT). PCR amplification products were quantitated, respectively, by using "Pico Green dsDNA Quantitation Kit" (trade name, product of Molecular Probes, Inc., OR, U.S.A.), and a solution containing the 16S rRNA gene at 6 ng/μL was prepared.

<Other conditions>

The base sequence of the fluorescence emitting probe was 5'CTAATCCTTT-(Texas Red)GGCGAT-(Dabcyl)AAATC3' in which the 9$^{th}$ T from the 5'end was modified with Texas Red and the 15$^{th}$

T from the 5'end was modified with Dabcyl. Modifications were conducted in a similar manner as in Example 7. In addition, the 3'end of the probe was phosphorylated to inhibit any extension from the 3'end. As a forward primer and a reverse primer, those employed in conventional PCR were used (E10F, E1400R) (namely, primers not modified with any fluorescent dye). As a real-time PCR apparatus, "iCycler" (trade mark, manufactured by Bio-Rad Laboratories, Inc., CA, U.S.A.) was used.

For both of the conventional PCR method and the real-time quantitative PCR method, the following PCR conditions were employed: 1st denature: 95℃, 120 seconds; denaturation: 95℃, 60 seconds; annealing: 56℃, 60 seconds; and extension: 72℃, 70 seconds. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively. As a Taq polymerase, "Gene Taq" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan) was used. The primers were each added at 100 nM in terms of final concentration in both of the conventional PCR method and the real-time quantitative PCR method. The DNA solution was used as a standard template solution, and was added at concentrations of from 0.6 pg to 6 ng/reaction, respectively. Using as a template an amplified product of a 16S rRNA gene derived from *Paracoccus denitfificans* DSM 413 as prepared in the above-described manner, the template was added to the reaction system to give concentrations of from

0.6 pg to 6 ng/reaction, respectively. The fluorescence emitting primer was added at 50 nM. Measurement of fluorescence was conducted once after denature and once after annealing in each cycle. The emitting rate of fluorescence (%) was determined in a similar manner as in Example 38.

2) Results

The results of real-time monitoring of the amplified products by the fluorescent emitting probes are shown in FIG. 35. It has been found from this diagram that amplified products can be monitored by using fluorescence emitting probes. Further, a relation between the number of cycles required to reach a threshold [log $F_n$ (emitting rate of flurescence, %) = 1.8] and the count of initially-added DNA is illustrated in FIG. 35. As is readily appreciated from this diagram, it is understood that the number of cycles and the number of copies added initially is in a linear relation. Incidentally, the correlation coefficient at this time was 0.9993 (R2 = 0.9993). Accordingly, it has been found from this diagram that the quantitation of initial copies of a target gene can be accurately achieved from the $n^{th}$ number of a cycle in which the threshold is reached.

From the above results, it has been proven that the determination of an initial concentration of a target nucleic acid (the number of copies of the target nucleic acid existed before amplification) is feasible by real-time quantitative PCR

making use of fluorescence emitting probes.

Example 40

(Detection of single nucleotide polymorphism by using a fluorescence emitting probe or a fluorescence quenching probe)

Based on a specific example, a description will be made about a method for detecting single nucleotide polymorphism from a denaturation curve by using a fluorescence emitting probe or fluorescence quenching probe.

1) Experimental procedures

As the fluorescence emitting probe, the same fluorescence emitting probe as that employed in Example 39 was used. As the fluorescence quenching probe, that having a similar base sequence as the fluorescence emitting probe and modified at the 5'end thereof with "BODIPY FL" was used {(BODIPY FL)-5'CTAATCCTTTGGCGATAAATC3'}. The modification was conducted in a similar manner as in Example 8. Employed as targets were a base sequence ((5')GATTTATCGC CAAAGGATTA G(3')), which was 100% complementary with above-described fluorescence emitting probe and fluorescence quenching probe, and a base sequence ((5')GATTTATCGT CAAAGGATTA G(3')) complementary with above-described fluorescence emitting probe and fluorescence quenching probe except for the inclusion of single nucleotide polymorphism that the $10^{th}$ C from the 5'end was replaced by T. The probe was added to a final concentration

of 100 nM. A synthesized target DNA was added to a final concentration of 400 nM. The composition of a hybridization solution was similar to that employed in Example 12. As the synthesized target DNA, one of two targets furnished for this experiment was used. The experiment was conducted by adding the solution, which had been prepared beforehand under the above-described conditions, into a fluorescence measuring tube and heating the solution at 0.1℃/sec from 30℃ to 80℃, during which measurement of fluorescence was continuously conducted.

From the results of this fluorescence measurement, probe-target denaturation curves were prepared. An evaluation was made as to whether or not a sequence including single nucleotide polymorphism can be discriminated from a difference in the denaturation curves. As an experimental apparatus, "iCycler" (trade mark, manufactured by Bio-Rad Laboratories, Inc., CA, U.S.A.) was employed. As fluorescence filters, a fluorescence filter for Texas Red provided by Bio-Rad Laboratories, Inc., CA, U.S.A. was used for the detection of fluorescence from the fluorescence emitting probe, and a fluorescence filter for FITC also provided by Bio-Rad Laboratories, Inc., CA, U.S.A. was employed for the detection of fluorescence from the fluorescence quenching probe.

2) Results

The results are diagrammatically shown in FIG. 36. It has been found from the diagram that for each of a fluorescence

emitting probe and a fluorescence quenching probe, the Tm value of its denaturation curve with a target containing single nucleotide polymorphism is lower by about 10℃ than the Tm value of its denaturation curve with a 100% complementary target. This indicates that the existence or non-existence of a hydrogen bond as much as one base appeared as the above difference in Tm. From the foregoing, it has been proven that single nucleotide polymorphism can be easily distinguished by using a fluorescence emitting probe or a fluorescence quenching probe.

Example 41

(DNA chip making use of fluorescence emitting probes)

Based on a specific example, a description will be made about a DNA chip making use of fluorescence emitting probes.

1) Experimental procedures

Fluorescence emitting probes of the base sequences shown in Table 10 were prepared. All of them are fragmentary base sequences of human CYP21 gene, and contain SNPs in their base sequences.

As probe names, the SNPs ID numbers allotted by the Whitehead Institute (http://waldo.wi.mit.edu/cvar_snps/) were used as were. The synthesis process was similar to that in Example 7 except for the following two matters. (1) To the 5'end, an amino linker was introduced by using "5'-Amino-Modifier C12" (trade name, product of Glen Research Corporation,

VA, U.S.A.). (2) Depending upon the base sequence of the probe, the modification with Texas Red was conducted using not only "Amino-Modifier C6 dT" but also "Amino-Modifier C6 dC" (trade name, product of Glen Research Corporation, VA, U.S.A.). The base sequences of the probes and the positions modified with Texas Red and Dabcyl in the probes are shown in Table 10. As target nucleic acids, those presented in Table 11 were used.

Table 10

Used Fluorescence Emitting Probes

| Probe name | Base sequence (SNPs at the underlined position) | Position modified by Texas Red as counted from the 5'end (5'end base: $0^{th}$) | Position of Dabcyl as counted from the 5'end |
|---|---|---|---|
| WIAF-10544 | 5'CGCAGCCGAG CATGGAAGA3' | 6 | 12 |
| WIAF-13038 | 5'CGCTGCTGCC CTCCGG3' | 5 | 11 |
| WIAF-10600 | 5'AAGGGCACGT GCACATGGC3' | 9 | 15 |
| WIAF-10579 | 5'CATCGTGGAG ATGCAGCTGA GG3' | 5 | 11 |
| WIAF-10578 | 5'CCTGCAGCAT CATCTGTTAC CTCAC3' | 10 | 16 |

Table 11

Base Sequences of Target Nucleic Acids

| Probe name | Base sequence | Remarks |
|---|---|---|
| No. 1 100% match target | 5'TCTTCCATGC TCGGCTGCG3' | Not modified |
| No. 1 1 mismatch target | 5'TCTTCCATGG TCGGCTGCG3' | Not modified; mismatched at the underlined position |
| No. 2 100% match target | 5'CCGGAGGGCA GCAGCG3' | Not modified |
| No. 2 1 mismatch target | 5'CCGGAGGACA GCAGCG3' | Not modified; mismatched at the underlined position |
| No. 3 100% match target | 5'GCCATGTGCA CGTGCCCTT3' | Not modified |
| No. 3 1 mismatch target | 5'GCCATGTGCA AGTGCCCTT3' | Not modified; mismatched at the underlined position |
| No. 4 100% mismatch target | 5'GCCTGCCACG AGGCTCTCC3' | Not modified |
| No. 4 1 mismatch target | 5'GCCTGCCACC AGGCTCTCC3' | Not modified; mismatched at the underlined position |
| No. 5 100% match target | 5'GTGAGGTAAC AGATGATGCT GCAGG3' | Not modified |
| No. 5 1 mismatch target | 5'GTGAGGTAAC AGTTGATGCT GCAGG3' | Not modified; mismatched at the underlined position |

<Preparation of DNA chip>

Spotting was conducted by applying one spot per probe solution. Except for this, a DNA chip was prepared in a similar manner as in the above-described preparation of the DNA chip making use of the fluorescence quenching probes.

In each of the probes of the present invention fixed on a slide glass, fluorescence from Texas Red is quenched when the probe is not hybridized with a target nucleic acid but, when hybridized, emission of fluorescence substantially increases compared with the emission of fluorescence when not hybridized.

<Detection or determination method of SNPs>

A 100% match target mixture solution - which contained the five 100% targets at concentrations of 100 μM, respectively, in 50 mM TE buffer (pH 7.2) - was placed on the DNA chip prepared as described above. A 1 mismatch target mixture solution - which contained the five 1 mismatch targets at concentrations of 100 μM, respectively - was prepared likewise, and was placed on a DNA chip which was different from the DNA chip on which the 100% match target mixture solution was placed. Cover glasses were placed over those solutions and were sealed with a nail varnish to avoid leakage of the target nucleic acids. Therefore, the two DNA chips were prepared in total in this test. Each of those chips was continuously observed for the emission of fluorescence at varied temperatures, and denaturation curves with the targets were prepared.

<Measuring Equipment>

Detecting or determining equipment was similar to that illustrated in FIG. 13.

2) Results of the experiment

The results of the experiment are illustrated in FIG. 37. It is understood from the diagram that in all the probes, the intensity of fluorescence increased as the temperature dropped. This indicates that each fluorescence emitting probe was hybridized with its corresponding target base sequence. It has therefore been demonstrated that a denaturation curve between a probe according to the present invention and a target nucleic acid can be easily monitored by the method of this invention. Further, the difference in Tm value between a probe which matches 100% with a target nucleic acid and a probe which mismatches by one base with the target nucleic acid was as much as about 10℃ in this investigation, so that it was possible to easily distinguish these probes from each other from their denaturation curves. Accordingly, this experiment has demonstrated that the use of a DNA chip according to the present invention makes it possible to simultaneously practice an analysis of plural types of SNPs.

Example 42

(Gene amplification and real-time detection of amplified products on a DNA chip on which fluorescence emitting probes and fluorescence quenched probes were

fixed)

Based on a specific example, a description will be made about a method for conducting gene amplification and also real-time monitoring of amplified products on a DNA chip with fluorescence emitting probes and florescence quenching probes fixed thereon. Further, detection of SNPs was conducted from denaturation curves between the amplified genes and the fluorescence emitting probes and fluorescence quenching probes.

1) Experimental procedures

(1) Fluorescence emitting probes

Fluorescence emitting probes and fluorescence quenching probes are shown in Table 12. They had the same base sequences as those employed in Example 41. The fluorescence emitting probes were used in a form phosphorylated at the 3'ends thereof. They were synthesized in a similar manner as in Example 41. The base sequences of the probes and the positions modified with Texas Red and Dabcyl in the probes are as indicated in Table 10.

(2) Fluorescence quenching probes

The base sequences of the fluorescence quenching probes are the same as those of the fluorescence emitting probes. To the 5'ends of the fluorescence quenching probes, an MMT amino linker was introduced using "5'-Amino-Modifier C12" (trade name, product of Glen Research Corporation, VA, U.S.A.). Subsequent

to deprotection of TFA as a protecting group, the respective oligonucleotides were modified with "BODIPY FL" (trade name, product of Molecular Probes, Inc., OR, U.S.A.) via the amino linker. The fluorescence quenching probes were in a form phosphorylated at the 3'ends thereof. As target nucleic acids, those shown in Table 11 were used. Except for these, details of their purification and modification procedures were similar to those practiced in Example 8.

(3) Primers

As a forward primer, one having the base sequence of 5'CTTGGGGGGGCATATCTG3' was used. As a reverse primer, on the other hand, one having the base sequence of 5'ACATCCGGCTTTGACTCTCTCT3' was employed. This primer set can amplify a section (2509 bp) of the human CYP21 gene. The fluorescence emitting probes and the fluorescence quenching probes have base sequences 100% complementary with their corresponding, SNPs-free amplified products. It was, therefore, expected that the intensities of fluorescence from the fluorescent emitting probes and fluorescence quenching probes shown in Table 12 would increase with the corresponding amplified products.

<Preparation of DNA chip>

On a slide glass, the individual probe solutions were spotted at a rate of one spot per probe solution. Except for this, a DNA chip was prepared in a similar manner as in the

preparation of the DNA chip making use of the quenching probes.

Where a fluorescence emitting probe is fixed on a slide glass, fluorescence from Texas Red is quenched when the probe is not hybridized with a target nucleic acid. When hybridized, however, the emission of fluorescence substantially increases compared with the emission of fluorescence when not hybridized. Where a fluorescence quenching probe is fixed on a slide glass, conversely, "BODIPY FL" emits fluorescence when not hybridized with a target nucleic acid but, when hybridized, significantly quenches fluorescence compared with the emission of fluorescence when not hybridized.

<Procedures of real-time monitoring PCR>

Using as a template the human genome employed in Example 28, PCR was conducted on the DNA chip while using the above-mentioned primers. PCR modification products were detected by the fixed fluorescence emitting probes or fluorescence emitting probes. The experiment was carried out using the equipment illustrated in FIG. 13. On the DNA chip with the fluorescence emitting probes and fluorescence emitting probes fixed thereon, a solution containing the primers, the template, Taq polymerase, dNTP, $MgCl_2$ and the like was placed. To avoid leakage of the solution, a cover glass was placed over the solution and sealed with a nail varnish. The chip was mounted on a transparent warming plate with a temperature control program stored therein, and a PCR reaction was conducted

on the chip. Amplified products were detected in real time by tracking changes in fluorescence from the fixed fluorescence emitting probes and fluorescence quenched probes by the microscope shown in FIG. 13.

The first denature was conducted at 95℃ for 120 seconds, followed by PCR cycles under the following conditions: denaturation: 95℃/60 sec, annealing: 69℃/60 sec, and extension: 72℃/120 sec. As primer concentrations, the concentrations of the forward primer and reverse primer were both set at 0.5 μM in terms of final concentration. The template was added at a final concentration of 1.5 ng/μL. As the DNA polymerase, "Gene TaqTM" (trade name, product of NIPPON GENE CO., LTD., Tokyo, Japan) was used at a concentration of 0.5 unit/20 μL. The concentration of Mg ions was set at 2 mM. dNTP was added to give a final concentration of 2.5 mM, respectively.

<Preparation of denaturation curves>

Denaturation curves between the fixed fluorescence emitting probes and fluorescence quenching probes and the PCR amplification products were prepared in a similar manner as in Example 41 to conduct detection of SNPs.

2) Results

The results of the experiment are shown in FIG. 38. It is understood from the diagram that in all the probes, the change in fluorescence increases with the number of cycle. It has, therefore, been demonstrated that gene amplification and

real-time detection of the amplified products can be conducted at the same time by the method of the present invention. The results of the preparation of the denaturation curves between the amplification products and the respective probes are shown in FIG. 39. It is appreciated from the diagram that in all the probes, a significant change in fluorescence was observed as the temperature became lower. This indicates that the fluorescence emitting probes and fluorescence quenching probes hybridized with the corresponding target base sequences. Accordingly, it has been ascertained to be possible to easily monitor the denaturation curves between the probes of the present invention and the target nucleic acids. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10600 are in substantial conformity with the denaturation curve between the artificially-synthesized, mismatch-free target and the WIAF-10600 probe as obtained in Example 41, thereby indicating that the human genome employed as a template in this Example is 100% complementary with the base sequence of the probe WIAF-10600. The denaturation curves between the amplification product and the fluorescence emitting probe and fluorescence quenching probe WIAF-10578 are in substantial conformity with the denaturation curve between the artificially-synthesized, mismatch-free target and the WIAF-10578 probe as obtained in Example 41, thereby indicating

that the human genome employed as a template in this Example contains a mismatch relative to the base sequence of the probe WIAF-105787. As can be appreciated from the foregoing, it has been found that use of a DNA chip according to the present invention makes it possible to simultaneously conduct an analysis of plural types of SNPs in an amplification product after a genetic amplification is conducted.

Table 12

Used Fluorescence Emitting Probes and Fluorescence Quenching Probes

| Probe name | Probe type | Sequence | Position modified by Texas Red as counted from the 5'end (5'end base: $0^{th}$) | Position of Dabcyl as counted from the 5'end |
|---|---|---|---|---|
| WIAF-10600-No. 1 | Fluorescence emitting probe | 5'AAGGGCACGT GCACATGGC3' | 6 | 12 |
| WIAF-10578-No. 2 | Fluorescence emitting probe | 5'CCTGCAGCAT CATCTGTTAC CTCAC3' | 5 | 11 |
| WIAF-10600-No. 3 | Fluorescence emitting probe | 5'AAGGGCACGT GCACATGGC3' | 9 | 15 |
| WIAF-10579-No. 4 | Fluorescence emitting probe | 5'CCTGCAGCAT CATCTGTTAC CTCAC3' | 5 | 11 |